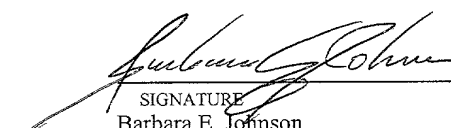


Form PTO-1390 (REV 10-95)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 702-000648	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (37 CFR 1.5) 09/509391	
INTERNATIONAL APPLICATION NO PCT/EP98/06183		INTERNATIONAL FILING DATE 25 September 1998 (25.09.98)		PRIORITY DATE CLAIMED (25.09.97) (01.05.98)	
TITLE OF INVENTION ISOLATED AND RECOMBINANT ANTIMICROBIAL PEPTIDES THROMBOCIDIN-1 (TC-1) AND THROMBOCIDIN-2 (TC-2) OR VARIANTS THEREOF					
APPLICANT(S) FOR DO/EO/US Jeroen KRIJGSVELD, Sebastianus Antonius Johannes ZAAT, Jacob DANKERT, Alma Johanna KUIJPERS, Gerardus Henricus Maria ENGBERS, and Jan FEIJEN					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.					
1 <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371					
2 <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.					
3 <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1)					
4 <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date					
5 <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))					
a <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).					
b <input type="checkbox"/> has been transmitted by the International Bureau					
c <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)					
6 <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2))					
7 <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))					
a <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau)					
b <input type="checkbox"/> have been transmitted by the International Bureau.					
c <input type="checkbox"/> have not been made, however, the time limit for making such amendments has NOT expired					
d <input checked="" type="checkbox"/> have not been made and will not be made.					
8 <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))					
9 <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))					
10 <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5))					
Items 11. to 16. below concern document(s) or information included:					
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98					
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included					
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment					
<input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.					
14. <input type="checkbox"/> A substitute specification					
15. <input type="checkbox"/> A change of power of attorney and/or address letter					
16. <input checked="" type="checkbox"/> Other items or information:					
a. WO 99/15548-Front Page with Abstract, specification, claims and drawings (37 pp.)					
b. Search Report (6 pp.)					
c. International Preliminary Examination Report completed 29.12.99 and Annexes (10 pp.)					

422 Rec'd PCT/PTO 27 MAR 2000

U S APPLICATION NO (if known, see 37 CFR 1.5) 09/509391		INTERNATIONAL APPLICATION NO PCT/EP98/06183		ATTORNEY'S DOCKET NUMBER 702-000648		
17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO..... \$840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) \$670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ... \$760.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$96.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY		
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00		
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE			
Total claims	14 - 20 =	0	X \$18.00	\$ 0		
Independent claims	2 - 3 =	0	X \$78.00	\$ 0		
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$ 0		
TOTAL OF ABOVE CALCULATIONS =				\$ 970.00		
Reduction of 1/2 for filing by small entity, if applicable Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28)				\$ 0		
SUBTOTAL =				\$ 970.00		
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))				\$ 0		
TOTAL NATIONAL FEE =				\$ 970.00		
Fee for recording the enclosed assignment (37 CFR 1.21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$ 0		
TOTAL FEES ENCLOSED =				\$ 970.00		
				Amount to be: refunded	\$	
				charged	\$	
a <input checked="" type="checkbox"/> A check in the amount of <u>\$970.00</u> to cover the above fees is enclosed. b <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. An original and two copies of this Transmittal Letter are enclosed c <input checked="" type="checkbox"/> The Assistant Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No <u>23-0650</u> . An original and two copies of this Transmittal Letter are enclosed NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status. SEND ALL CORRESPONDENCE TO: Barbara E. Johnson Webb Ziesenheim Logsdon Orkin & Hanson, P.C. 700 Koppers Building 436 Seventh Avenue Pittsburgh, Pennsylvania 15219-1818 Telephone: (412) 471-8815 Facsimile: (412) 471-4094						
				 SIGNATURE Barbara E. Johnson NAME 31,198 REGISTRATION NUMBER		

PATENT APPLICATION/PCT
Attorney Docket 702-000648

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :

JEROEN KRIJGSVELD, : ISOLATED AND RECOMBINANT
SEBASTIANUS A.J. ZAAT, : ANTIMICROBIAL PEPTIDES
JACOB DANKERT, : THROMBOCIDIN-1 (TC-1)
ALMA JOHANNA KUIJPERS, : AND THROMBOCIDIN-2 (TC-2)
GERARDUS H.M. ENGBERS : OR VARIANTS THEREOF
and JAN FEIJEN :

International Application :
No. PCT/EP98/06183 :

International Filing Date :
25 September 1998 :

Priority Dates Claimed :
25 September 1997 :
01 May 1998 :

Serial No. Not Yet Assigned :

Filed Concurrently Herewith :

Pittsburgh, Pennsylvania
March 27, 2000

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington DC 20231

Sir:

Prior to initial examination, please amend the above-
identified patent application as follows:

IN THE CLAIMS:

Original claims 1-20 were amended during Chapter II proceedings by substituting new claims 1-21 which were received by the International Bureau on June 22, 1999. Please cancel original claims 1-20, cancel amended claims 1-21 and rewrite them as new claims 22-32, and add new claims 33-35 as follows:

--22. Antimicrobial peptides having a specific arrangement and disulfide-linkage of four cystein residues.

23. The antimicrobial peptides according to claim 22 wherein the peptides have a three-dimensional structure as shown in figure 13.

24. The antimicrobial peptides according to claim 22 wherein the peptides are CXC chemokines.

25. The antimicrobial peptides according to claim 22 wherein the peptides are thrombocidin-1 (TC-1), or variants thereof, which comprise at least in part the sequence as shown in figure 1A, indicated by the label TC-1, and have antimicrobial
5 activity.

26. The antimicrobial peptides according to claim 22 wherein the peptides are thrombocidin-2 (TC-2), or variants thereof, which comprise at least in part the sequence as shown in figure 1A, indicated by the label TC-2, and have antimicrobial
5 activity.

27. The antimicrobial peptides, or variants thereof, according to claim 22 wherein said peptides, or variants thereof, are prepared recombinantly.

28. The antimicrobial peptides, or variants thereof, according to claim 22 wherein said peptides, or variants thereof, exhibit antibacterial activity against gram-positive and gram-negative bacteria, for example *Escherichia coli*, *Bacillus*
5 *subtilis*, *Streptococcus sanguis*, *Streptococcus pneumoniae*, *Staphylococcus epidermis*, and *Staphylococcus aureus*.

29. The antimicrobial peptides, or variants thereof, according to claim 22 wherein said peptides, or variants thereof, exhibit antifungal activity against fungi, for example *Candida albicans*, *C. glabrata*, *Cryptococcus neoformans*, *Aspergillus*
5 *flavus*, *A. fumigatus*, and *Pseudoallescheria spec.*

30. The antimicrobial peptides, or variants thereof, according to claim 22 containing an additional N-terminal Histag sequence and having an enhanced antimicrobial activity in comparison to the same peptides without N-terminal Histag.

31. The antimicrobial peptides, or variants thereof, according to claim 22 for use in the treatment of bacterial infections in humans and animals.

32. The antimicrobial peptides, or variants thereof, according to claim 31 wherein the bacterial infection is endocarditis.

33. A method of minimizing or treating a fungal infection in a human or animal in need of such treatment, comprising administering in unit dosage form at least one peptide according to claim 22 together with at least one pharmaceutically
5 acceptable excipient.

34. The method according to claim 33 wherein said fungal infection is endocarditis.

35. The method according to claim 33 wherein said peptide contains a Histag sequence and has enhanced antimicrobial activity in comparison to the same peptide without a Histag sequence.--

IN THE ABSTRACT:

After the claims, please insert a page containing the Abstract Of The Disclosure, which is attached hereto as a separately typed page.

REMARKS

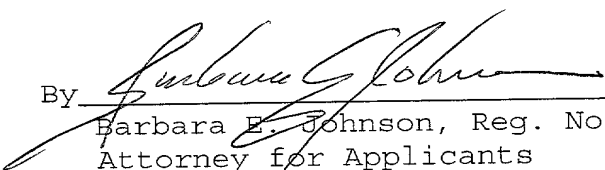
The above claim amendments are being made to bring the pending claims in alignment with customary U.S. practice.

Examination and allowance of pending claims 22-35 are respectfully requested.

Respectfully submitted,

WEBB ZIESENHEIM LOGSDON
ORKIN & HANSON, P.C.

By


Barbara E. Johnson, Reg. No. 31,198
Attorney for Applicants
700 Koppers Building
436 Seventh Avenue
Pittsburgh, PA 15219-1818
Telephone: 412/471-8815
Facsimile: 412/471-4094

ISOLATED AND RECOMBINANT ANTIMICROBIAL PEPTIDES
THROMBOCIDIN-1 (TC-1) AND THROMBOCIDIN-2
(TC-2) OR VARIANTS THEREOF

ABSTRACT

The present invention relates to isolated and recombinant antimicrobial peptides thrombocidin-1 (TC-1) and thrombocidin-2 (TC-2), or variants thereof, which comprise at least in part the sequence as shown in figure 1 indicated by the label TC-1 and TC-2, and have antimicrobial activity against gram-positive and gram-negative bacteria, for example *Escherichia coli*, *Bacillus subtilis*, *Streptococcus sanguis*, *Streptococcus pneumoniae*, *Staphylococcus epidermis*, and *Staphylococcus aureus*, and/or against fungi, for example *Candida albicans*, *C. glabrata*, *Cryptococcus neoformans*, *Aspergillus flavus*, *A. fumigatus*, and *Pseudoallescheria spec.* The invention further relates to the use of said peptides, or variants thereof, for the preparation of a medicament for the treatment of bacterial or fungal infections, such as endocarditis, in humans and animals and the use of said peptides, or variants thereof, in release systems for prevention of bacterial or fungal infections in humans and animals.

ISOLATED AND RECOMBINANT ANTIMICROBIAL PEPTIDES
THROMBOCIDIN-1 (TC-1) AND THROMBOCIDIN-2 (TC-2) OR
VARIANTS THEREOF

The present invention relates to isolated antimicrobial peptides TC-1 and TC-2 or variants thereof and to recombinantly prepared TC-1 and TC-2 or variants thereof.

- 5 Antibiotics are commonly used in the treatment and/or prevention of infectious diseases caused by various microorganisms. However, resistance of bacteria against these antibiotics often occurs. Thus, methicillin-resistant Staphylococcus aureus (MRSA) and
10 methicillin-resistant Staphylococcus epidermis (MRSE) are well-known resistant microorganisms, among other bacterial species, responsible for serious nosocomial infections that are very difficult to treat.

- Conventional antibiotics kill bacteria by
15 binding to specific targets that are involved in bacterial DNA and protein synthesis or in cell wand synthesis. Resistance can occur when bacteria modify these targets, such that antibiotics do not bind to these proteins, or when bacteria produce specific enzymes that
20 inactivate the antibiotics. Glycopeptide antibiotics such as vancomycin can be used to treat these resistant microorganisms, but their use must be limited to prevent development of resistance to these "kill or cure" remedies as well.

- 25 As more and more bacteria become resistant to routinely applied antibiotics, there is an increasingly urgent need for alternative antibacterial agents.

- The object of the present invention is therefore to provide new antimicrobial agents against
30 which microorganisms do not rapidly become resistant.

 This object is achieved by the invention by providing new, isolated or recombinant, antimicrobial peptides thrombocidin-1 (TC-1) and thrombocidin-2 (TC-2),

or variants thereof, such as TC-1', which comprise, at least in part, the sequence as shown in figure 1 and have broad antimicrobial activity. These peptides, or variants thereof, thus may be effectively used as antibiotics in the treatment of several infectious diseases. These peptides can be isolated from both human and animal tissue.

"Variants" of isolated or recombinant peptides TC-1 and TC-2 are peptides that are at least 70% homologous, preferably at least 80%, more preferably at least 90%, most preferably at least 95%, to TC-1 and TC-2 and also have antimicrobial activity in vitro, such as TC-1'.

It has been found that human and animal blood platelets contain factors that exhibit antibacterial and antifungal activity in vitro. Upon thrombin-activation platelets are known to release lysozyme, as well as a number of other cationic peptides. A number of these platelet-derived peptides have been identified, such as platelet factor-4 (PF-4), RANTES, connective tissue activating peptide (CTAP-III), platelet basic protein (PBP), and neutrophil activating peptide (NAP-2).

In the research that led to the invention, new peptides have been isolated from platelet granules, and have been purified and characterized. These peptides are small and strongly positively charged proteins and are named thrombocidins (TC). The positive charge of thrombocidins presumably accounts for their antibacterial activity. Thus, like other cationic antibacterial proteins, thrombocidins most likely form pores in bacterial membranes, as a result of which these bacteria will die. Since thrombocidins act on the bacterial membrane itself which can not easily be modified, resistance will not rapidly occur.

Thrombocidins are stored in alpha-granules of platelets and are released following thrombin-activation. The most active thrombocidins, TC-1 and TC-2 and variants, such as TC-1', have been isolated, purified and

characterized as described in further detail in the following examples. In short, thrombocidins were isolated from a large batch of human platelets. The first step was to isolate the platelet granules in which the

5 thrombocidins are present. These platelet granules subsequently were disrupted and the protein content was collected for further purification. The thrombocidins were separated from other proteins on the basis of molecular size, polarity and charge.

10 The new peptides of the invention appear to be derivatives of NAP-2 and CTAP-III. NAP-2 itself is a N-terminal cleavage product of CTAP-III. TC-1 has been shown to be a mixture of C-terminal truncation products of NAP-2, of which the 7436 Da peptide, lacking two C-
15 terminal amino acids, is the main component (referred to as variant TC-1'; figure 1, table 1). A form of NAP-2 with an additional N-terminal tyrosine was also present as a minor component. TC-2 has been identified as a C-terminal truncation product of CTAP-III lacking the last two C-
20 terminal amino acids, with a molecular weight of 9100 (figure 1A, table 1). Thrombocidins identified thus far are indicated in fig. 1A, together with the known sequences of CTAP-III and NAP-2 (fig. 1).

As described earlier, the thrombocidins are C-
25 terminal truncation products of NAP-2 and CTAP-III, both of which are chemokines of the CXC family. These chemokines have a specific arrangement and disulfide-linkage of 4 cystein residues, giving the molecule a characteristic three dimensional structure (fig 13).
30 Therefore it is well possible that other chemokines or chemokine-like molecules possess antimicrobial activity like the thrombocidins.

Based on the amino acid sequence of the native proteins the DNA coding for TC-1, NAP-2 and CTAP-III were
35 now cloned and used to recombinantly produce these proteins in order to obtain larger quantities. Hereto, coding DNA was amplified from a human bone marrow cDNA library by PCR and cloned into a NdeI/BamHI digested

pET9a or pET16b expression vector. Constructs were transformed to E. coli BL21DE3LysS cells and gene expression was induced by adding IPTG to growing cultures. Bacteria were harvested, lysed in guanidine, and recombinant proteins were purified to homogeneity in a two-step purification. Sequences of the purified recombinant proteins are shown in figure 2. Recombinant thrombocidins were also produced in the milk of animals.

Several classes of antibacterial proteins contain disulfide bonds. As far as it has been investigated, the presence of these disulfide bonds has been found to be essential for antibacterial activity of HNP-2 (Selsted and Harwig, 1989), GNCPs (Yomogida et al, 1995) and beneficial for activity of protegrins (Harwig et al, 1996). Because disulfide formation is critical for antibacterial activity, a prokaryotic system is not an obvious way to produce these proteins recombinantly. HNP has been produced recombinantly in E. coli, but indeed this product had no antibacterial activity, probably due to misfolding of the protein (Piers et al, 1993). Chemokines like NAP-2, CTAP-III (Proudfoot et al, 1997) and IL-8 (Lindley et al, 1988) have been produced in E. coli, but these proteins had to be refolded after they had been purified, a procedure which was not needed to observe antibacterial activity of the recombinant thrombocidins.

Bactericidal activity of TC-1' and TC-2 has been confirmed against a number of gram-positive, such as Streptococcus sanguis, Staphylococcus aureus, Staphylococcus epidermidis, and Bacillus subtilis and gram-negative bacteria such as Escherichia coli, which are found in a wide variety of infections.

One example of an infection in which the peptides of the invention may be used is endocarditis. Endocarditis is a serious infectious heart disease with high morbidity and mortality, associated with abnormalities of the heart endothelium or the heart valves. Lesions due to these abnormalities give rise to

platelet adherence and activation. Together with other blood proteins, these platelets form a dense meshwork covering the lesion, to which bacteria that have entered the blood stream will adhere. In turn, more platelets will adhere to the bacteria and the 'clot' will grow, ultimately causing malfunctioning of the valve resulting in the need for valve replacement. S. sanguis is one of the microorganism prevalent in native valve endocarditis. Other bacterial, or fungal microorganisms may, however, also be found.

In the platelet clot, bacteria are protected from phagocytic cells, which cannot penetrate the dense platelet meshwork. In contrast, platelet-derived thrombocidins are capable of penetrating the clot and thus may effectively prevent bacterial proliferation.

Fungi against which the peptides of the invention may be effective comprise, for example: Candida albicans, C. glabrata, Cryptococcus neoformans, Aspergillus flavus, A. fumigatus, and Pseudoallescheria spec.

The present invention thus provides new, isolated or recombinantly prepared peptides TC-1 and TC-2, or variants thereof, such as TC-1* (fig 1 and 2), which exhibit antibacterial and/or antifungal activity and can be used in the treatment of infections in humans and animals. Furthermore, the peptides, or variants thereof, of the present invention can be used for the preparation of a medicament for the treatment of bacterial and/or fungal infections.

The invention is further illustrated, but not limited by the following examples and figures.

EXAMPLES**EXAMPLE 1****Isolation, purification and characterization of TC-1 and****5 TC-2****A. Isolation of granule protein from thrombocytes**

10 Buffy coats of human blood from healthy subjects were obtained from the Central Laboratory for Bloodtransfusion, Amsterdam, The Netherlands. Eight buffy coats were pooled in a transfer bag (NPBI, Emmer-Compascuum, The Netherlands) (ca. 550 ml), and 200 ml of PBS + 0.38% tri-sodium citrate (w/v) was added. The bag was blown tight with air and centrifuged for 5 min at 600 g and 20°C. The upper phase, containing mainly platelets, 15 was transferred to a new transfer bag. To this platelet concentrate, 1/9 volume of citrate solution was added (75 mM trisodium citrate; 38 mM citric acid). The bag was blown tight again, and was centrifuged for 10 min at 1750 g (20°C) to pellet platelets. Platelets were resuspended 20 in the same bag in Tris-citrate (63 mM Tris-HCl; 95 mM NaCl; 5 mM KCl; 5mM EDTA; pH 6.8) by gentle massage, and kept shaking overnight at 22°C. Then, platelets were collected in a siliconized flask and the transfer bag was washed with Tris-citrate. Processing of 48 buffy coats 25 routinely yielded ca. 75 ml of highly concentrated platelet suspension containing <0.05% residual leukocytes.

To isolate platelet granules, the platelet concentrate was cavitated three times under nitrogen at 30 60 atm in a Parr cavitation chamber, and cavitate was collected in siliconized 50 ml tubes (Falcon). This resulted in ca. 90% homogenization of the platelets as determined by Coulter counting. Intact platelets and platelet ghosts were removed by centrifuging the cavitate 35 at 5000 g for 20 min. The supernatant was collected and centrifuged at 12000 g for 20 min, yielding the granules in the pellet. The pellet was resuspended in 5% acetic acid, and sonicated for 30 seconds (pulsed) on ice to

rupture granules. The sonicate was kept at 4°C for 24 hours, and subsequently was centrifuged at 125000 g. The supernatant containing granule protein was dialyzed against 5% acetic acid.

5

B. Purification of thrombocidin-1 and -2

A rapid two-step purification protocol was developed for the purification of TC-1 and TC-2 from platelet granule protein: i) cation exchange
10 chromatography, and ii) preparative acid urea polyacrylamide gel chromatography (AU-PAGE), to yield highly purified protein preparations.

i) Cation exchange chromatography

As an ion exchange matrix, CM-sepharose 25
15 (Pharmacia) was used; phosphate buffer (50 mM, pH 7.0) was used as the mobile phase. A 25 ml sample, containing 3.5 mg/ml of granule protein from approximately 40 buffy coats, was loaded at 0.8 ml/min. Subsequently, the column was washed with phosphate buffer, and protein was eluted
20 with a salt gradient from 0 to 1 M NaCl. Fractions were collected, dialyzed and assayed for the presence of antibacterial proteins by running two separate acid urea gels in parallel. One gel was silverstained (fig 3), the other gel was used in an overlay assay to detect
25 antibacterial activity (fig 4). E. coli ML35 was used as the test organism. Selected fractions were analyzed by tricine gel electrophoresis to estimate molecular weights of the (partially) purified protein (fig 5). The activity present in the starting material (fig 3, cav) is eluted
30 in fractions 35 through 75. Major antibacterial activity can be assigned to two proteins, the most cationic protein is designated as thrombocidin-1 (TC-1), the slightly less cationic as thrombocidin-2 (TC-2). These proteins migrate in an SDS gel as proteins with an
35 apparent molecular weight of 5.5 and 6.5 kD, respectively (fig 5).

ii) Continuous AU-PAGE

Fractions eluted from the CM-sepharose column containing antibacterial protein (30 through 75) were pooled, lyophilized, and subjected to a second
5 purification step utilizing continuous gel electrophoresis. Cylindrical gels (3,7x6 cm, 12.5% acrylamide, 3M urea, 5% acetic acid) were poured in a model 491 Prep Cell (BioRad, Veenendaal, The Netherlands) and polymerized at 37°C. Prerunning was at 200V for 2 h.
10 Sample (max. 450 µl) was electrophorized at 40 mA. Protein was eluted in 5% acetic acid at 1 ml/min and collected in 5 ml fractions. Again, fractions were analyzed in two urea gels run in parallel, followed by staining or by an assay for antibacterial activity (fig
15 6). TC-1 and TC-2 could effectively be separated (fig 6a). Both proteins had considerable activity against E. coli ML35 (fig 6b). Purified TC-1 and TC-2 were lyophilized and redissolved in 0,01% acetic acid, and stored at -20°C until further analysis.

20

C. Structure of thrombocidin-1 and -2

Bactericidal proteins thrombocidin-1 and -2, purified from human blood platelets, were analyzed by MALDI and electrospray (ES) mass spectrometry. ES
25 analysis of TC-1 (fig 7a) yielded a molecular weight of $7436,3 \pm 1,3$ Da. Analysis by MALDI (fig 8) also revealed a peak of this size, next to an additional number of peaks with M+1 of 7107.2, 7227.7 and 7602,0 Da. The molecular weights of these proteins can be explained by
30 assuming that these proteins are C-terminal truncation products of NAP-2; the calculated molecular weights correspond well with the values experimentally determined (Table 1). These data suggest that TC-1 is a mixture of C-terminally truncated forms of NAP-2. The 7436 Da
35 protein seems to be the main component. We designated this protein TC-1'.

ES spectroscopy of TC-2 (fig 7b) yielded a molecular weight of $9100,5 \pm 1,3$. This value was

confirmed by MALDI-tof spectroscopy. In addition to TC-2, only one minor contamination was present (10081 Da, fig 9). Partial sequencing of TC-2 indicated that the N-terminus of TC-2 is identical to that of CTAP-III. Based on the mass-spectrometrical data (figs 7b and 9) however, it appears that the mass found experimentally was smaller than the mass of CTAP-III (table 1). This can be explained by assuming that TC-2 is truncated C-terminally and misses 2 amino acids compared to CTAP-III.

10 Thrombocidins identified thus far are indicated in fig 1, together with the sequences of CTAP-III and NAP-2.

Table 1. Interpretation of mass-spectrometrical data of TC-1 and TC-2: comparison with CTAP-III

Component	Mol. weight (Da)		Sequence of	
	MALDI/ES	Calc	N-terminus	C-terminus
CTAP-III		9287,2	NLAKGKEESLDSPLYAELR....	AGDESAD
TC-1a	7106,2	7105,8		AELR....AG
TC-1b	7226,7	7220,9		AELR....AGD
TC-1*	7436,3	7437,5		AELR....AGDES
TC-1d	7601,0	7600,7		YAEELR....AGDES
TC-2	9100,5	9101,6	NLAKGKEESLDSPLYAELR....	AGDES

EXAMPLE 2Production of recombinant (r)CTAP-III, rNAP-2, rTC-1, rTC-1' and rTC-2.

From a human bone marrow CDNA library

5 (Clontech, Palo Alto, USA) DNA coding for PBP was amplified in a PCR. 5'TATAGGATCCATGAGCCTCAGACTTGATACCACC-3' and 5'TATAGGATCCTCAATCAGCAGATTCATCACCTGCCAAT-3' were used as forward and reverse primers, respectively. BamHI restriction sites (underlined) were added to allow

10 cloning in a suitable vector. A stop sequence (boldface) was included to allow proper transcription termination at the stage of protein expression. This PCR was performed using 2 ng of template DNA and Pfu DNA polymerase, which has proofreading capacity. The resulting product was of

15 the expected size (400 bp). This product served as a template in a second PCR to produce the coding DNA of TC-1, TC-2, CTAP-III, NAP-2 and TC-1', a variant of TC-1 which lacks two C-terminal amino acids (Ala-Asp) and carries two additional N-terminal amino acids (Ala-Glu)

20 (fig 2). These PCR products were cloned into expression vectors. For CTAP-III, NAP-2 and TC-1 the reverse primer was the same as the reverse primer described above. The forward primers were as follows:

25

for CTAP-III and TC-2:

5'GTGTAACATATGAACTTGGCGAAAGGCAAAGAG-3';

for NAP-2 and TC-1':

5'GTGTAACATATGTATGCTGAACTCCGCTGCATG-3';

5 and for TC-1:

5'GTGTAACATATGTATCTCCGCTGCATGTGTATAAAG-3'.

NdeI restriction sites (underlined) were included to allow cloning into the vectors. The PCR products were digested with NdeI/BamHI and ligated into a pET9a (TC-2, CTAP-III and TC-1') or a pET16b vector (NAP-2 and TC-1), linearized with NdeI and BamHI. The recombinant proteins produced using the pET9a vector carry an additional N-terminal methionine, and are therefore designated as rMTC-1' and rMTC-2 and rMCTAP. The recombinant proteins produced using the pET16 vector carry an N-terminal His-tag, plus a tyrosine (Y) residue, and therefore designated as rYTC-1 and rYNAP. Constructs were transformed to E. coli DH5 α , and plated on LB/kanamycin (50 μ g/ml) (pET9a) or LB/ampicillin (50 μ g/ml) (pET16b) plates. Sequencing of cloned DNA confirmed the correct sequence in the constructs. Plasmids containing the correct insert were isolated and transformed to BL21(DE3)lyse cells, and plated on LB plates supplemented with the proper antibiotics. Of each plate, single colonies were picked, grown and stored in glycerol broth at 70°C until further use. Cultures of BL21(lyse) cells transformed with the CTAP-III, NAP-2 TC-1 or TC-1' genes containing pET expression vector were grown in LB medium supplemented with the proper antibiotics. Growing cultures with OD₆₆₀ of 0.3 were induced with IPTG (1mM final concentration). After 3 hours of induction, cells were harvested by centrifugation (5 min, 5000 g) and lysed in 20mM Tris HCl, pH 8,2 containing 6M guanidine HCl. Cell debris was removed by centrifugation. Supernatants of rMCTAP and r-MTC producing cells were dialyzed against 50mM phosphate buffer, pH 7,0.

rMTC-1*, rMTC-2 and rMCTAP were purified by CM-sepharose cationexchange chromatography and continuous acid urea gel electrophoresis, as described for TC-1* and TC-2 in example 1. The N-terminal His-tag in rYTC-1 and rYNAP allowed purification of these proteins using a His binding resin (Novagen). Final purification was performed by continuous AU PAGE. The structures of the purified recombinant proteins were confirmed by MALDI and ES mass-spectrometry.

10

EXAMPLE 3

Antibacterial activity

The experimental set-up for testing antibacterial activity of thrombocidins was as follows.

15 Bacteria from blood agar plates were grown overnight in tryptic soy broth (TSB), subcultured in fresh TSB and grown to log-phase in 2-3 hours. Bacteria were pelleted, washed once in 10 mM phosphate buffer (pH 7,0) + 1% TSB (v/v) and resuspended in the same medium to an OD₆₂₀ of

20 0,1. This suspension was further diluted 200 (B. subtilis) or 500 times (E. coli and S. aureus) to obtain suspensions containing 0,5-1 x 10⁵ colony forming units (cfu)/ml. In a polypropylene microtiter plate a serial dilution series of the protein to be tested was prepared

25 in 0,01% acetic acid. To 5 µl of every sample, 45 µl of bacterial suspension (0,5-1 x 10⁵ cfu/ml) was added. The plate was incubated on a rotary shaker (400 rpm) at 37°C. After 2 hours, 0,5 and 10 µl samples were plated on blood agar plates. Bactericidal activity was calculated the

30 next day after colony counting. All experiments were performed in duplicate.

Bactericidal activity of TC-1* and TC-2 was determined against E. coli ML35, S. aureus 42D and B. subtilis ATCC6633 in killing assays (fig 10).

35 In fig 10 it can be seen that TC-1* and TC-2 are bactericidal against a all three bacteria tested, and that TC-1* is the more active component.

Bactericidal activity of TC-2 was tested against a panel of other bacterial species. The same method was used as described above, except that 5% Brain Heart Infusion (BHI) (v/v in water) instead of phosphate buffer + TSB was used as a test medium and one concentration of TC-2 was tested, being 100 µg/ml. Bacteria tested were E. coli ML35, wild-type E. coli, S. aureus 42D, multi-resistant S. aureus (MRSA), multiresistant S. epidermis (MRSE), and S. sanguis J30 (fig 11). The MRSA and MRSE are as susceptible to TC-2 as S. aureus 42D, while S. sanguis J30 seems to be slightly less susceptible.

Analysis of TC-1 and TC-2 in a non-reducing tricine gel revealed that the migration of both peptides was retarded compared to their reduced forms. This indicated that TC-1 and TC-2 contain disulfide bridges. To investigate whether the disruption of these disulfide bridges influenced antibacterial activity, reduced TC-2, reduced and alkylated TC-2, and non-reduced TC-2 were analysed for antibacterial activity using an acid urea gel overlay system. All three forms of TC-2 were equally active (fig. 12), indicating that disulfide bridges are not needed for TC-2 antibacterial activity. TC-1 was treated in the same way and showed similar results.

In general, disulfide bonds of cationic antibacterial proteins are considered to be essential for their antibacterial activity (Selsted and Harwig, 1989, Yomogida et al, 1995, Harwig et al, 1996, Piers et al, 1993, Proudfoot et al, 1997 and Lindley et al, 1988). The fact that in TC-1 and TC-2 the disulfide bonds are not needed for antibacterial activity is an unexpected finding.

MBCs of rMTC-1*, rMTC-2 and rMCTAP were determined for a number of organisms (Table 2). It appeared that rMTC-1* and rMTC-2 are bactericidal for *B. subtilis*, although MBCs are somewhat higher than for the native proteins (fig 10). The MBC of rMTC-1* for *E. coli* (3,8 µM) is the same as the MBC of the native protein TC-

1* (fig 10), whereas the MBC of rMTC-1* for *S. aureus* (15 μ M) is approximately 2-fold higher than for TC-1* (fig 10). In contrast to this, rMCTAP was not bactericidal for *E. coli*, *B. subtilis* and *S. aureus* at concentrations up to 40 μ M. Recombinant NAP-2 (obtained from Bachem, Switzerland) was tested against *B. subtilis* up to 7 μ M, but no killing was observed.

10 Table 2. MBCs (μ M) of recombinant proteins for various bacteria.

	<u><i>B. subtilis</i></u>	<u><i>E. coli</i></u>	<u><i>S. aureus</i></u>
15 rMTC-1*	3,8	3,8	15
rMTC-2	7,5	15	>15
rMCTAP	>40	>30	>30
rNAP-2	>7		

Bactericidal activity of rYTC-1 has been tested in a liquid assay as has been done for the native TCs. In comparison to the native thrombocidins TC-1* and TC-2, rYTC-1 is equally active against *B. subtilis*, but is more active against *S. aureus* 42D, and *E. coli* ML35 (table 3, fig 10). Additional experiments showed that rYTC-1 is highly active against a large number of bacterial species and against *Cryptococcus neoformans* (Table 3).

Table 3. Microbial activity of rYTC-1

Organism	MBC (μ M) of rYTC-1
<u>E. coli</u> 69187 (EPEC)	0,9
<u>E. coli</u> 72540 (EPEC)	0,4
<u>E. coli</u> (genta 0)	0,9
<u>E. coli</u> ML35	0,9
<u>Pseudomonas aeruginosa</u>	3,8
<u>Neisseria meningitidis</u> W135	1,9
<u>Klebsiella</u>	1,9
<u>Bacillus subtilis</u> ATCC6633	0,4
<u>S. aureus</u> 42 D	1,9
MRSA	1,9
<u>S. Epidermis</u> RP62	0,9
<u>S. Epidermis</u> AMC 43	0,6
<u>S. Epidermis</u> AMC 48	0,6
<u>S. Epidermis</u> AMC 77	0,6
<u>S. Epidermis</u> AMC 82	1,2
<u>S. Epidermis</u> AMC 89	0,6
MRSE	1,2
<u>S. sanguis</u> U108	1,9
<u>S. sanguis</u> J30	1,9
<u>Cryptococcus neoformans</u>	0,4

Bactericidal activity of rMTC-1* against S. aureus, E. coli, and B. subtilis was significantly lower than that of rYTC-1 (table 2 and 3). The marked difference in bactericidal activity between rMTC-1* vs
5 rNAP-2, and between rMTC-2 vs rMCTAP (Table 3) shows that the 2 additional C-terminal amino acids, alanin (A) and aspartic acid (D) present in rNAP-2 and rMCTAP strongly reduce bactericidal activity. Although these C-terminal amino acids are also present in rYTC-1, this protein is
10 more active than rMTC-1*, which lacks the 2 C-terminal amino acids. In fact, rYTC-1 had more potent antibacterial activity than any of the native or recombinant proteins and rYTC-1 was also more active (MBC 0.4 μ M) against Cryptococcus neoformans than rMTC-1* (MBC
15 7.5 μ M) and rMTC-2 (MBC 15 μ M). This indicates, that the N-terminal His-tag-containing sequence of rYTC-1 is involved in enhanced bactericidal activity, which has not been demonstrated before.

FIGURE LEGENDS

Figure 1: Sequences of thrombocidins and related proteins.

5

Figure 2: Recombinant proteins produced. Boxes indicate antimicrobial activity enhancing sequence (Histag).

10

Figure 3: Analysis of CM-sepharose purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by silverstaining.

cav: crude granule extract (cavitate), starting material
15 for the purification.

Figure 4: Analysis of CM-sepharose purified platelet granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by
20 an overlay using E. coli as a test organism.
cav: crude granular extract (cavitate), starting material for the purification.

Figure 5: Analysis of CM-sepharose purified
25 platelet granular protein by tricine SDS-electrophoresis. Silverstained gel. Selected fractions (compare fig. 3) were analyzed.

Figure 6: Analysis of AU-PAGE purified platelet
30 granular antibacterial protein. Selected fractions (as indicated) were run on AU-gels followed by silverstaining (A) or followed by an overlay using E. coli as a test organism (B).
cav: crude granular extract (cavitate); CM: pooled active
35 fractions (30-75) eluted from a CM-sepharose column.

Figure 7: Electrospray mass spectrometrical analyses of TC-1 (A) and TC-2 (B).

Figure 8: MALDI-tof mass spectrometrical analysis of TC-1.

Figure 9: MALDI-tof mass spectrometrical analysis of TC-2.

10 Figure 10: Antibacterial activity of TC-1 (top panel) and TC-2 (bottom panel) against E. coli ML35, S. aureus 42D, and B. subtilis ATCC6633 ($0,5-1 \times 10^5$ cfu/ml) were incubated in the presence of serially diluted TC (concentrations are indicated). After 2 hours of
15 incubation bacteria were plated and survival was determined by colony counting. Medium: 10 mM phosphate buffer pH 7,0 + 1% TSB. Of TC-2 0,3 and 0,7 μ M were not tested against S. aureus and E. coli.

20 Figure 11: Killing of bacteria ($1-2 \times 10^5$ cfu/ml) by TC-2 after 2 hrs incubation.

Figure 12: Antibacterial activity of TC-2 and reduced TC-2. Panel A: silverstained acid urea gel. Panel
25 B: Overlay of acid urea gel (test organism: E. coli). Lanes 1: TC-2; Lanes 2: reduced and carboxymethylated TC-2. Lanes 3: TC-2 reduced by β -mercaptoethanol treatment. Each lane contains approximately 3 μ g of protein.

30 Figure 13: Three-dimensional structure of CXC chemokines.

REFERENCES

- SUBSTITUTE SHEET (RULE 26)**

AMENDED CLAIMS

1. Microbicidal peptides having a specific arrangement and disulfide-linkage of four cystein residues.

2. Microbicidal peptides according to claim 1
5 characterized in that the peptides have a three-dimensional structure as shown in figure 13.

3. Microbicidal peptides according to claim 1 characterized in that the peptides are CXC chemokines.

4. Microbicidal peptides according to claim 1
10 characterized in that the peptides are thrombocidin-1 (TC-1), or variants thereof which comprise at least in part the sequence as shown in figure 1, indicated by the label TC-1, and have microbicidal activity.

5. Microbicidal peptides according to claim 1
15 characterized in that the peptides are thrombocidin-2 (TC-2), or variants thereof which comprise at least in part the sequence as shown in figure 1, indicated by the label TC-2, and have microbicidal activity.

6. Peptides, or variants thereof, according to
20 claims 1-5 characterized in that said peptides, or variants thereof, are prepared recombinantly.

7. Peptides, or variants thereof, according to claims 1-6 characterized in that said peptides, or variants thereof, exhibit bactericidal activity against
25 gram-positive and gram-negative bacteria, for example Escherichia coli, Bacillus subtilis, Streptococcus sanguis, Streptococcus pneumoniae, Staphylococcus epidermis, and Staphylococcus aureus.

8. Peptides, or variants thereof, according to
30 claims 1-6 characterized in that said peptides or variants thereof exhibit fungicidal activity against fungi, for example Candida albicans, C. glabrata, Cryptococcus neoformans, Aspergillus flavus, A. fumigatus, and Pseudoallescheria spec..

9. Peptides, or variants thereof, according to
35 any of claims 1-8 characterized in that the variants of

the peptides are at least 70% homologous, preferably at least 80%, more preferably at least 90%, most preferably at least 95%, to said peptides and have microbicidal activity.

5 10. Peptides, or variants thereof, according to any of the preceding claims containing an additional N-terminal Histag sequence and having an enhanced microbicidal activity in comparison to the same peptides without N-terminal Histag.

10 11. Peptides, or variants thereof, according to any of the preceding claims for use in the treatment of bacterial infections in human and animals.

15 12. Peptides, or variants thereof, according to claim 11 **characterized in that** the bacterial infection is endocarditis.

13. Use of peptides, or variants thereof, according to any of the preceding claims for the preparation of a medicament for the treatment of bacterial infections in human and animals.

20 14. Use of peptides, or variants thereof, according to any of the claims 1-12 in release systems for prevention of bacterial infections in human and animals.

25 15. Use of peptides, or variants thereof, according to claim 13 or 14 **characterized in that** the bacterial infections is endocarditis.

16. Peptides, or variants thereof, according to any of the claims 1-12 for use in the treatment of fungal infections in human and animals.

30 17. Peptides, or variants thereof, according to claim 16 **characterized in that** the fungal infection is endocarditis.

18. Use of peptides, or variants thereof, according to any of the preceding claims for the preparation of a medicament for the treatment of fungal infections in human and animals.

19. Use of peptides, or variants thereof, according to any of the preceding claims in release

systems for prevention of fungal infection in human and animals.

20. Use of peptides, or variants thereof,
according to claim 18 or 19 ~~characterized in that~~ the
5 fungal infection is endocarditis.

21. Use of a Histag sequence for the
preparation of microbicidal peptides having an enhanced
microbicidal activity in comparison to the same peptides
without additional Histag sequence.

10

ART 34 AMDT

1/14

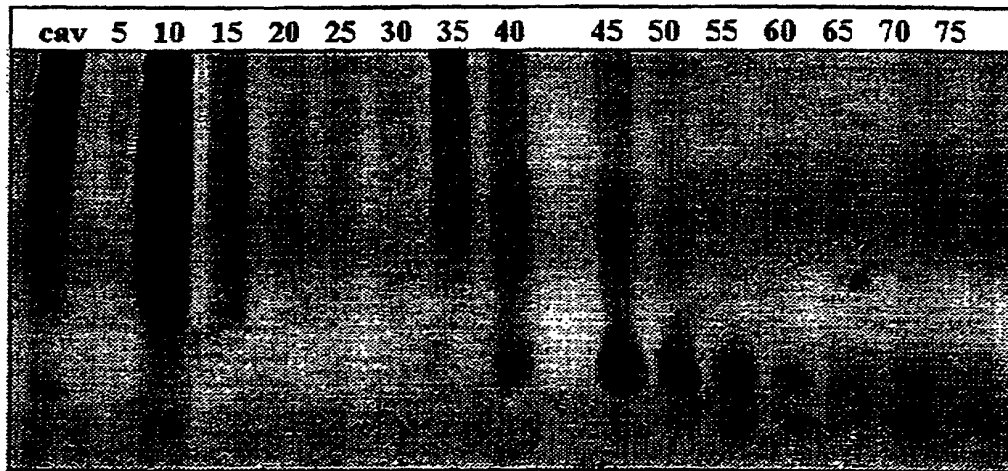
TC-1: LRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DESAD
 TC-1*: AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DES
 TC-2: NLAGG KEESL DSDLY AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DES
 CTAP-III: NLAGG KEESL DSDLY AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DESAD
 NAP-2: AELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DESAD

FIG. 1

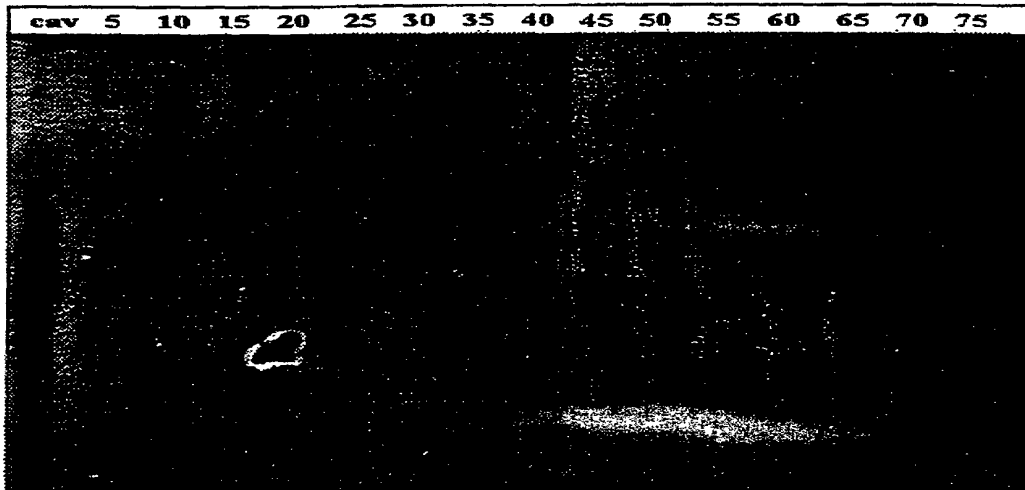
TC-1*: MAELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DES
 TC-2: MNLAGGKEESLSDLYAELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DES
 TC-1: MGHHHHHHHHHSSGHIEGRHM YLRMCICKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DESAD
 YNAP: MGHHHHHHHHHSSGHIEGRHM YAE LRMCICKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DESAI
 TC-2: MNLAGGKEESLSDLYAELRC MCIKT TSGIH PKNIQ SLEVI GKGTH CNQVE VIATL KDGRK ICLDP DAPRI KKIVQ KKLAGE DESAD

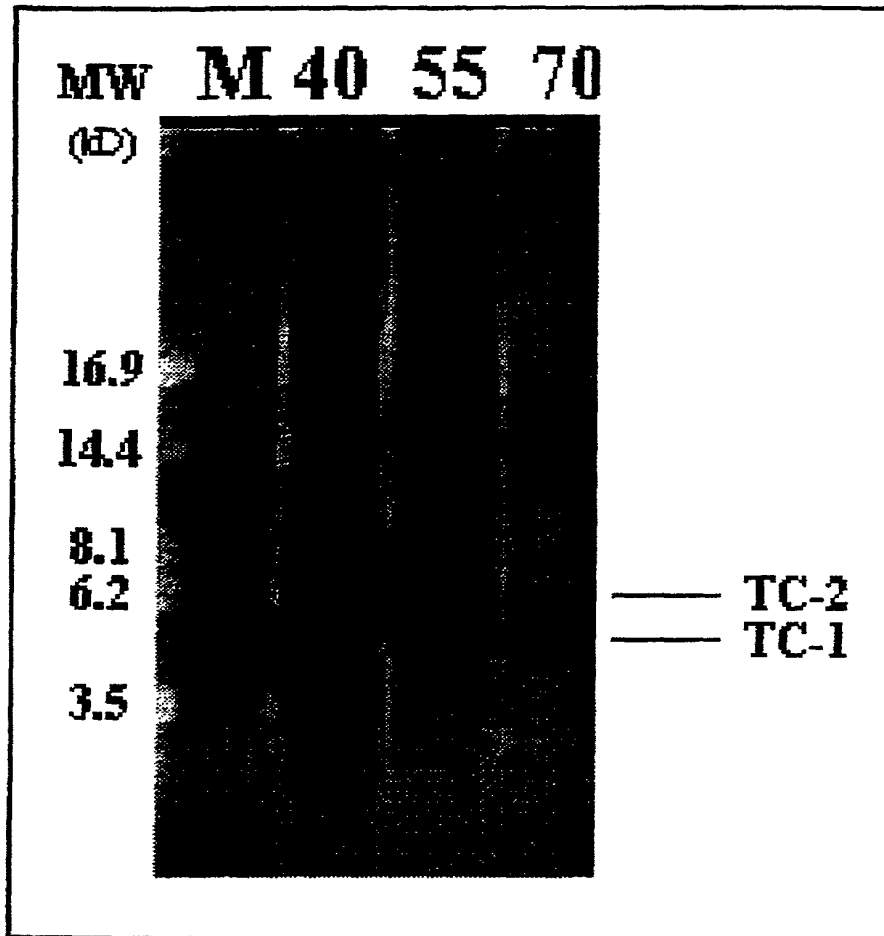
FIG. 2

2/14

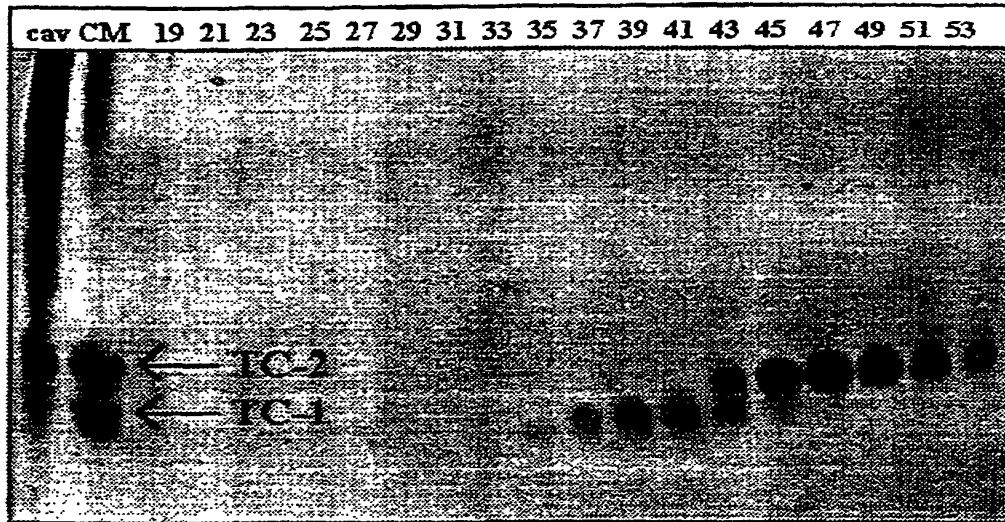
FIG. 3

3/14

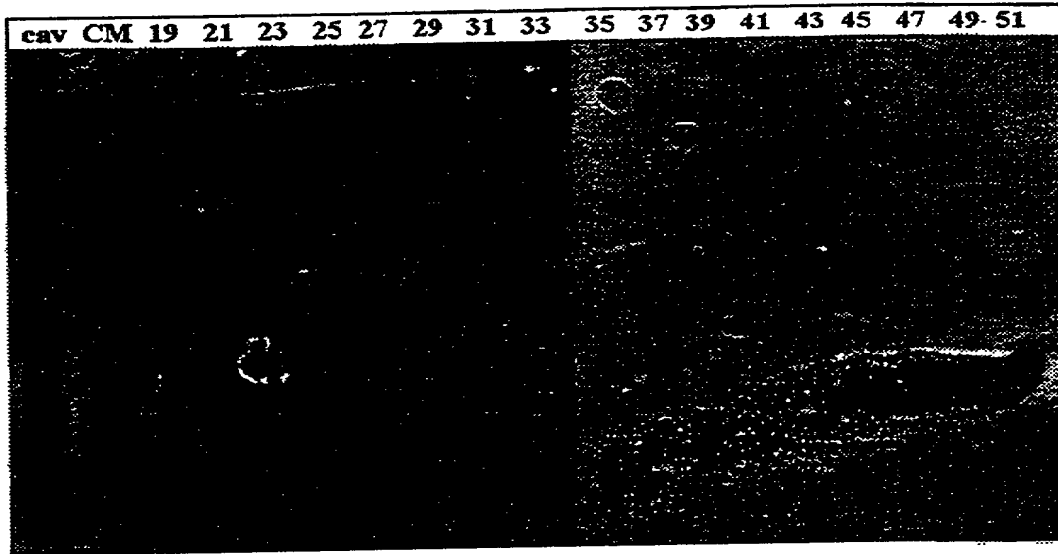
FIG. 4

FIG. 5

5/14

FIG. 6A

6/14

FIG. 6B

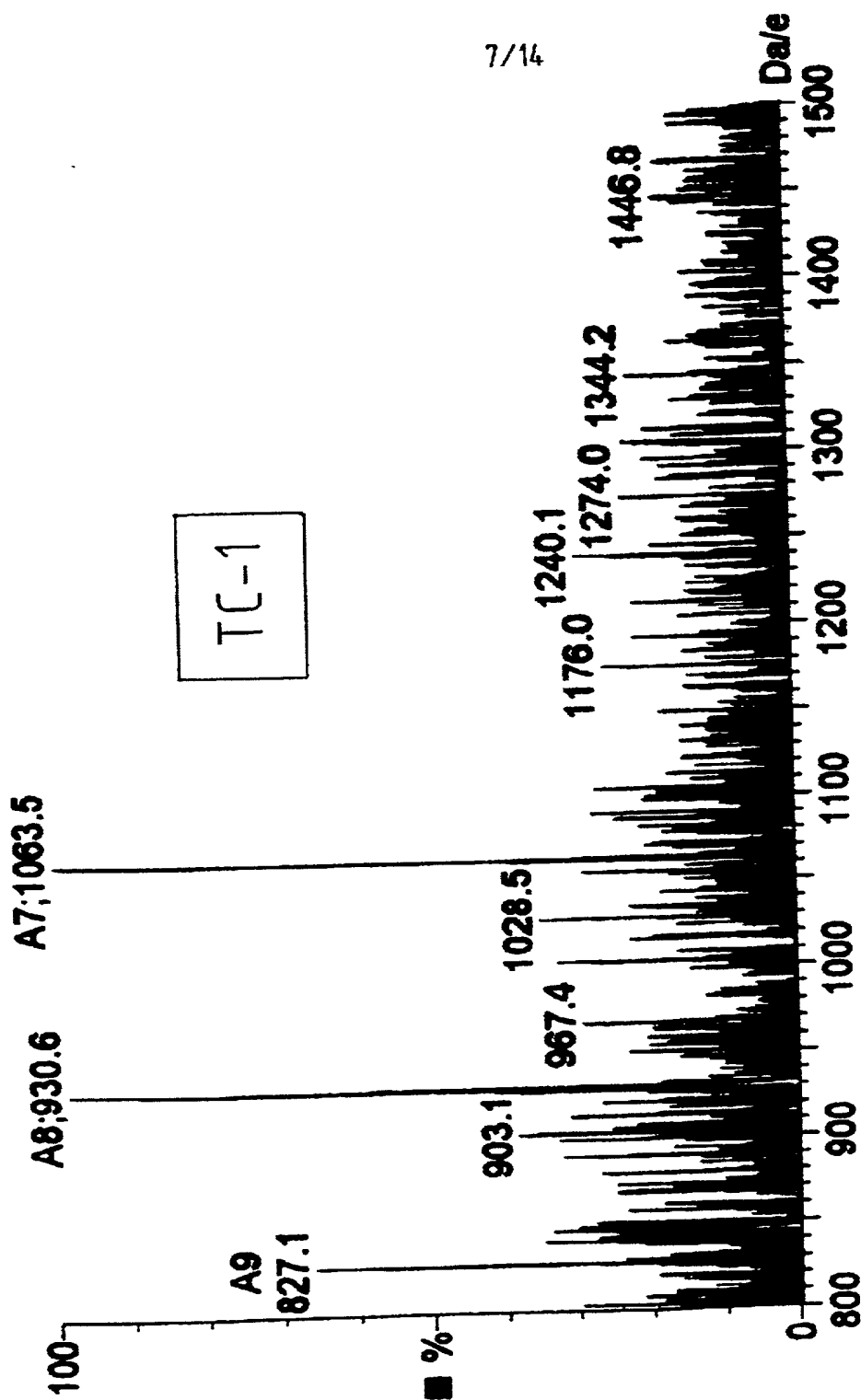


FIG. 7A

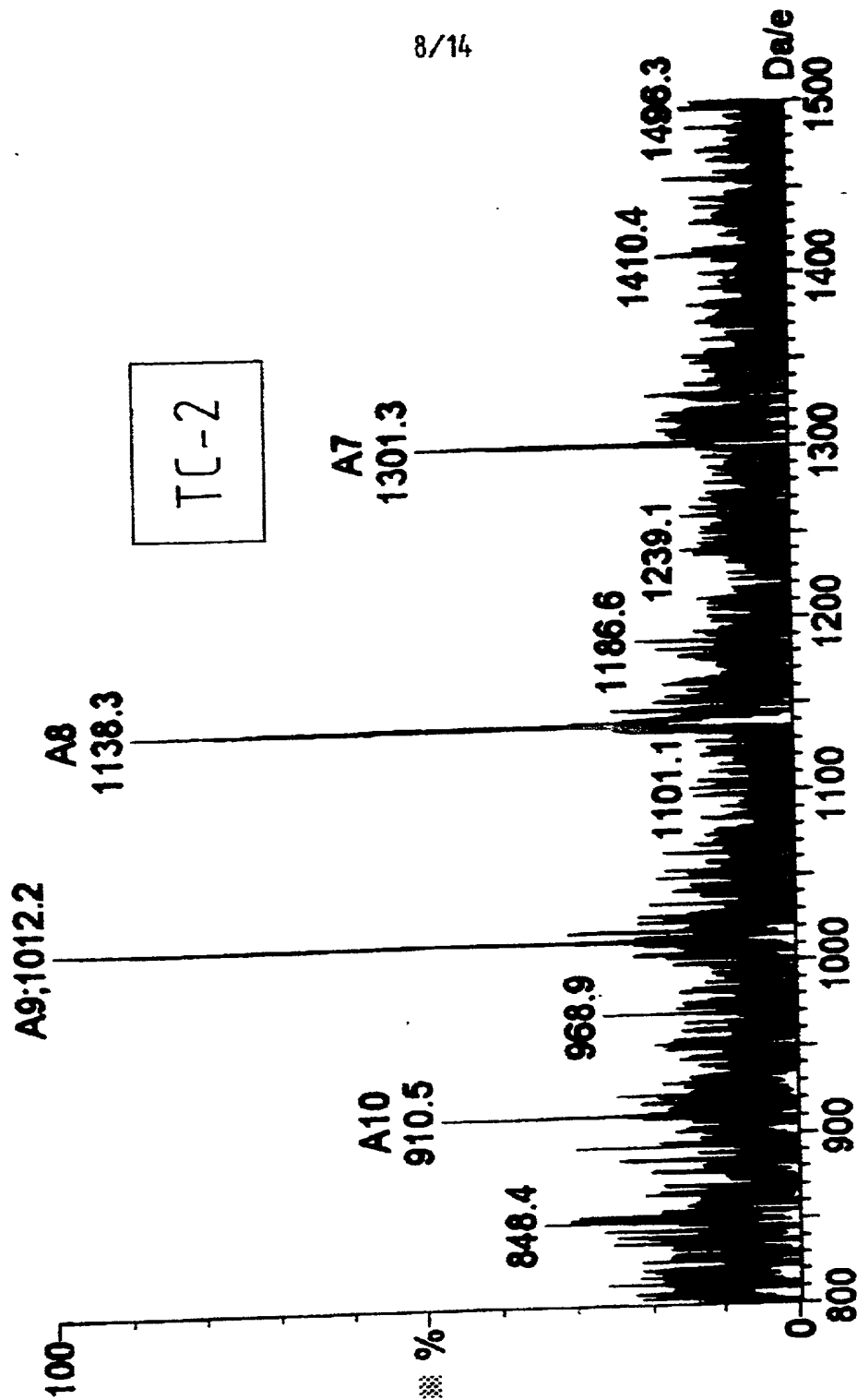


FIG. 7B

9/14

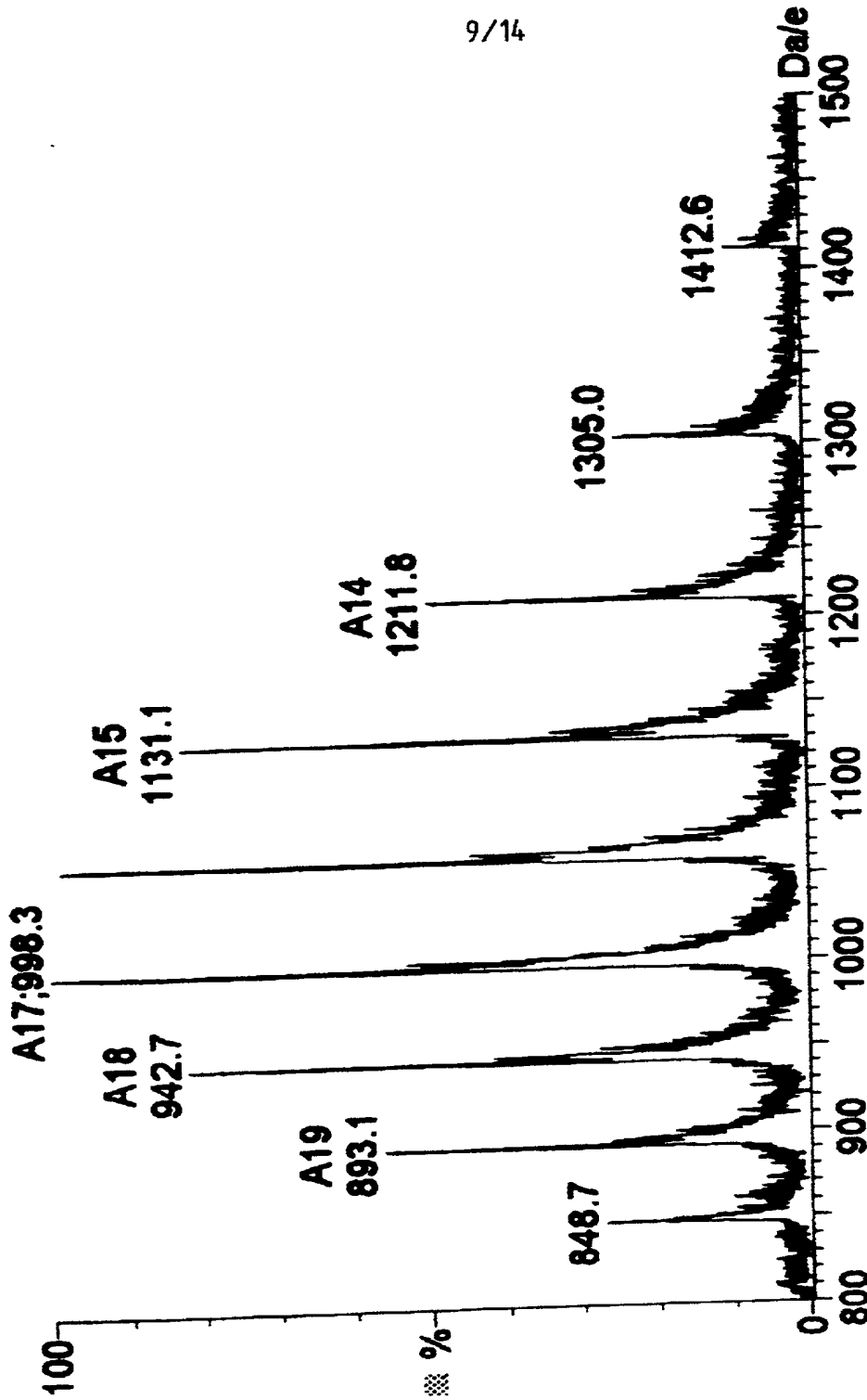
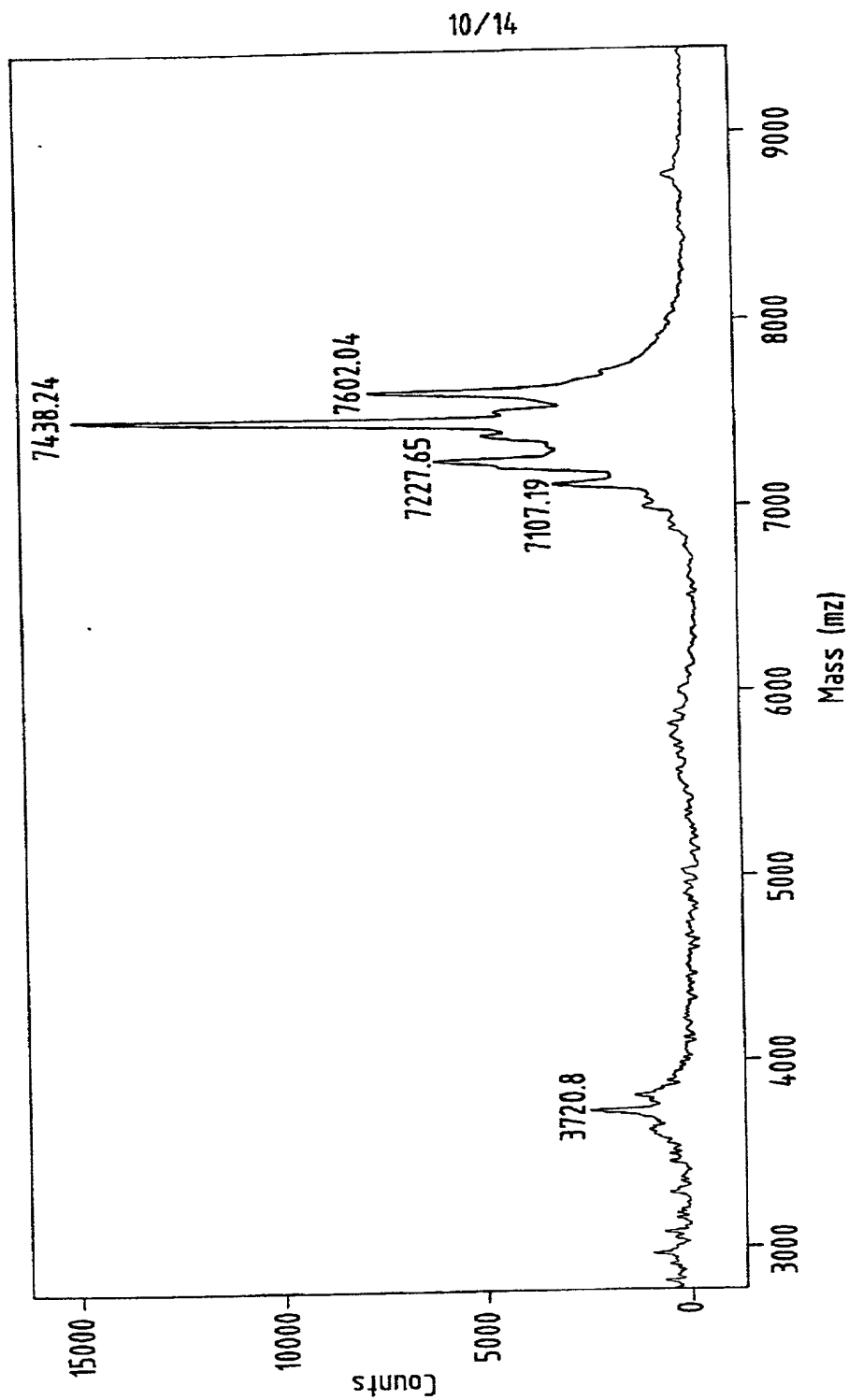
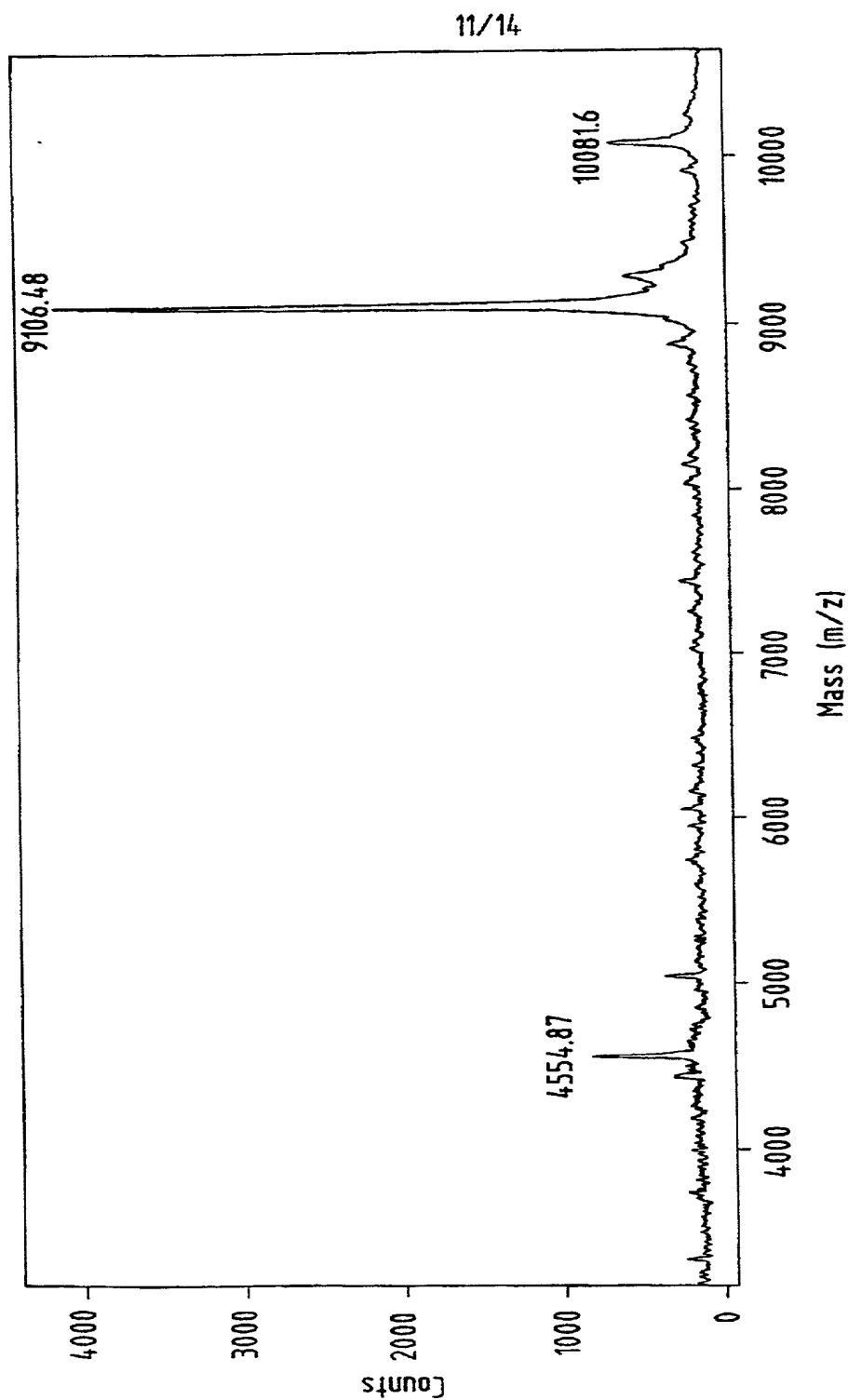


FIG. 7C

FIG. 8

FIG. 9

12/14

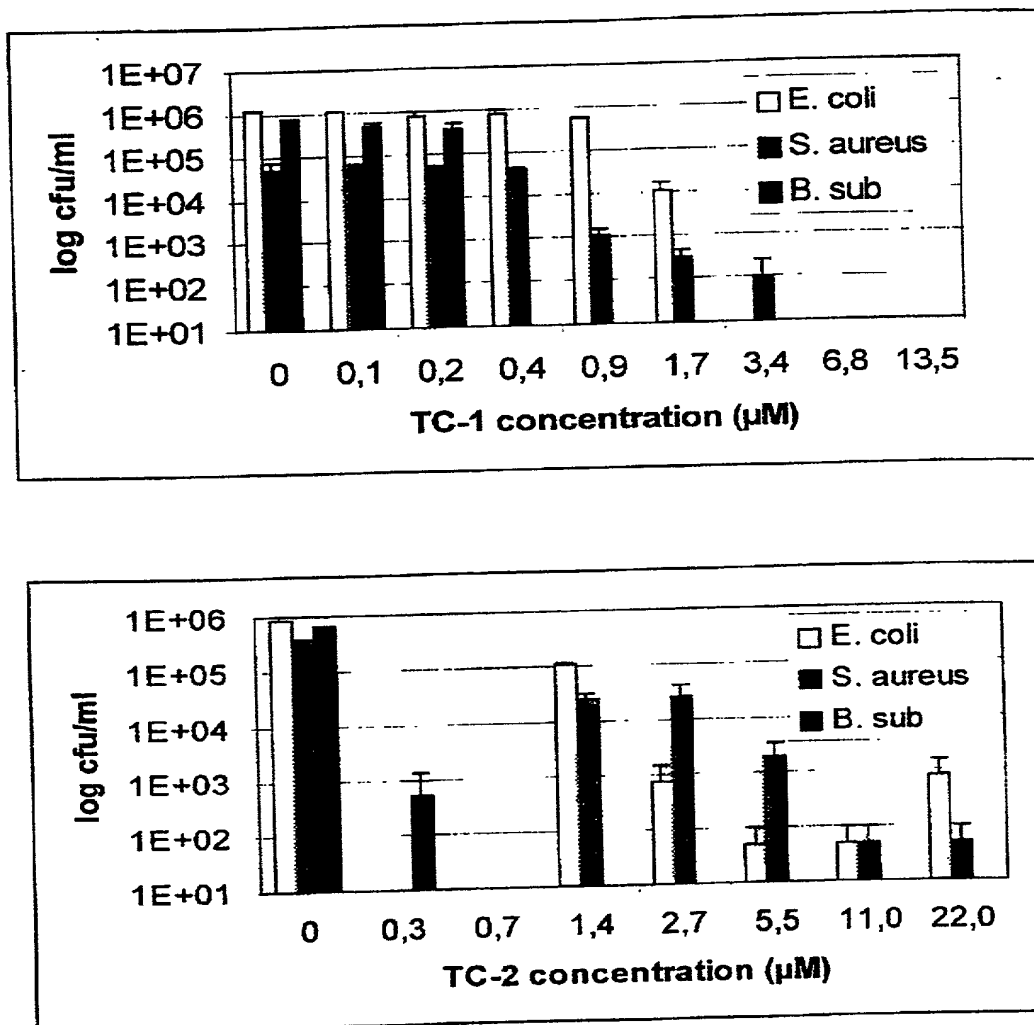
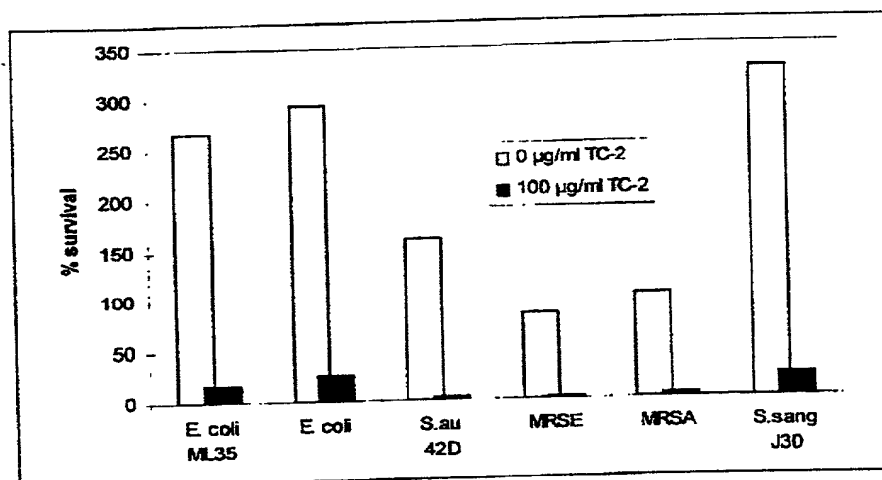
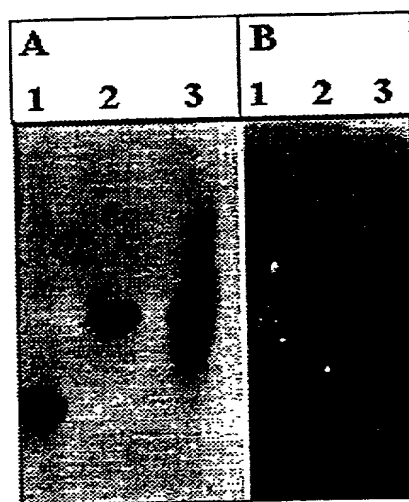
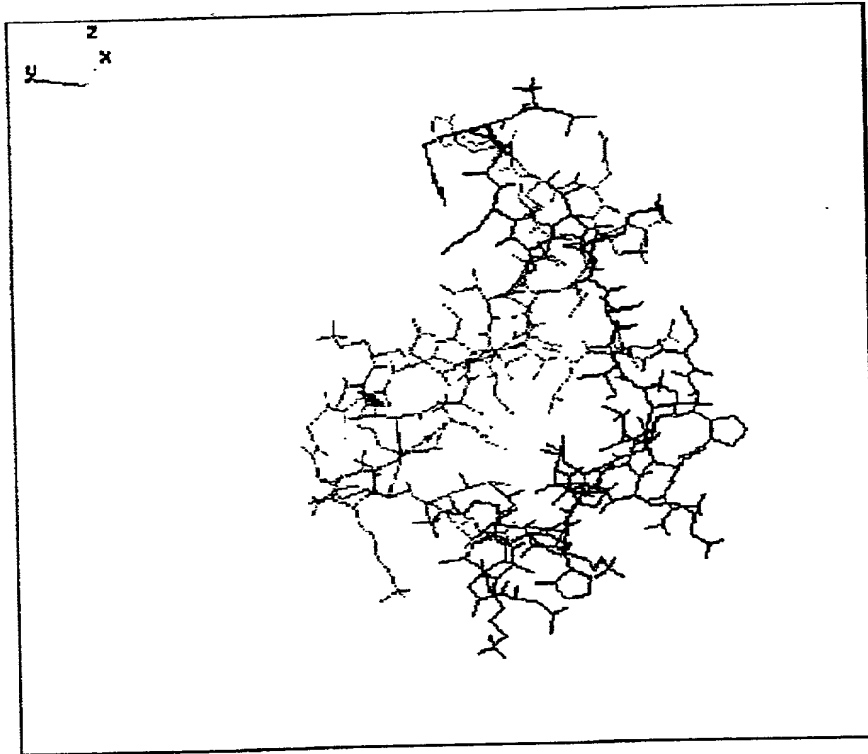


FIG. 10

13/14

FIG. 11FIG. 12

14/14

FIG. 13

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Isolated and recombinant antimicrobial peptides thrombocidin-1 (TC-1) and thrombocidin-2 (TC-2) or variants thereof, the specification of which

(check one)

☐ is attached hereto.

☒ was filed on September 25, 1998 as PCT/EP98/06183 as
Application Serial No. 09/509,391 received on March 27, 2000
and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
<u>97202934.2</u> (Number)	<u>Europe</u> (Country)	<u>25 September 1997</u> (Day/Month/Year Filed)	<input checked="" type="checkbox"/> Yes	<input type="checkbox"/> No
<u>98201411.0</u> (Number)	<u>Europe</u> (Country)	<u>1 May 1998</u> (Day/Month/Year Filed)	<input checked="" type="checkbox"/> Yes	<input type="checkbox"/> No
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/> Yes	<input type="checkbox"/> No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

William H. Logsdon	22,132	Paul M. Reznick	33,059	Jesse A. Hirshman	40,016
Russell D. Orkin	25,363	John W. McIlvaine	34,219	James G. Porcelli	33,757
David C. Hanson	23,024	Michael I. Shamos	30,424	Kent E. Baldauf, Jr.	36,082
Richard L. Byrne	28,498	Blynn L. Shideler	35,034	Christian Schuster	43,908
Frederick B. Ziesenheim	19,438	Julie W. Meder	36,216	Dean E. Geibel	42,570
Kent E. Baldauf	25,826	Lester N. Fortney	38,141	Thomas J. Clinton	40,561
Barbara E. Johnson	31,198	Randall A. Notzen	36,882	Nathan J. Prepelka	43,016

Send Correspondence to:

Barbara E. Johnson, 700 Koppers Building, 436 Seventh Avenue, Pittsburgh PA 15219-1818

Direct Telephone calls to: (name and telephone number) Barbara E. Johnson (412) 471-8815

Full name of sole or first inventor

KRIJGSVELD, Jeroen

Inventor's signature

Date 3 July 2000

Residence

Amsterdam, The Netherlands NLX

Citizenship

the Netherlands

Post Office Address

Blasiusstraat 130/1, NL-1091 CZ Amsterdam, The Netherlands

Full name of second joint inventor, if any

Zaat, Sebastianus Antonius Johannes

Second inventor's signature

Date 3 July 2000

Residence

Amstelveen, The Netherlands NLX

Citizenship

The Netherlands

Post Office Address

Burgemeester Haspelslaan 352, NL-1181 NG Amstelveen, The Netherlands

(Supply similar information and signature for third and subsequent joint inventors.)

300 Full name of third joint inventor, if any
DANKERT, Jacob

Third Inventor's signature

Date 3 July 2000

Residence

Baambrugge, The Netherlands NLX

Citizenship

The Netherlands

Post Office Address

Dorpsstraat 27, NL-1396 KG Baambrugge, The Netherlands

400 Full name of fourth joint inventor, if any

KUIJPERS, Alma Johanna

Fourth inventor's signature

Date 3 July 2000

Residence

Enschede, The Netherlands NLX

Citizenship

The Netherlands

Post Office Address

Breugelmansgaarde 134, NL-7511 PT Enschede, The Netherlands

500 Full name of fifth joint inventor, if any

LENGBERS, Gerardus Henricus Maria

Fifth Inventor's signature

Date 3 July 2000

Residence

Oldenzaal, The Netherlands NLX

Citizenship

the Netherlands

Post Office Address

Vlaanderenlaan 3, NL-7577 MB Oldenzaal, The Netherlands

600 Full name of sixth joint inventor, if any

FEIJEN, Jan

Sixth inventor's signature

Date 3 July 2000

Residence

Hengelo, The Netherlands NLX

Citizenship

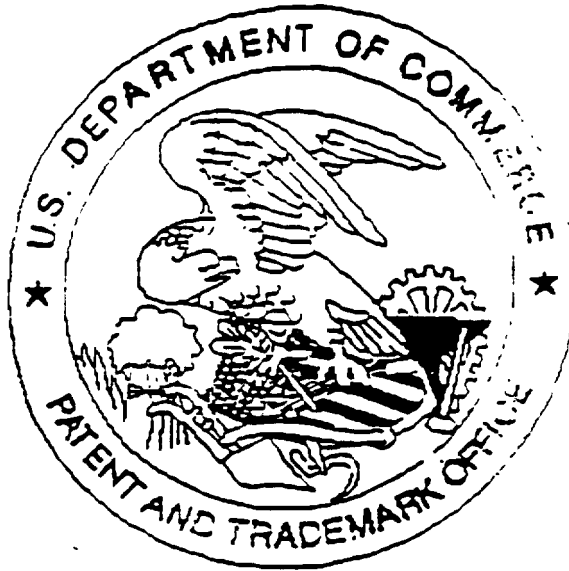
The Netherlands

Post Office Address

Oude Grensweg 96, NL-7552 GD Hengelo, The Netherlands

(Supply similar information and signature for subsequent joint inventors.)

United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies were found during scanning:

☐ Page(s) _____ of _____ were not present:
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present:
for scanning. (Document title)

☒ Scanned copy is best available. *Drawings*